R+D 7186 CH-01

Identification of bacteria.

The Gram staining procedure is well established for separation of large groups of bacteria, i.e. into Gram positive and Gram negative ones. This procedure was developed for microscopic purposes and is thus applied on bacteria attached to slides. Other staining procedures for separation of large groups of bacteria may be based upon binding of various dye-labeled compounds to specific parts of the bacterial wall. Thus, an alternate Gram staining procedure is based upon a fluorescence-labeled lectin which binds specifically to *N*-acetylglucosamine in the outer peptidoglycan layer of Gram positive bacteria (Sizemore R.K. *et al.* Applied and Environmental Microbiology, 1990). The peptidoglycan layer of Gram negative bacteria is covered by a membrane which is not labeled by this lectin. This can be used to distinguish between Gram positive and Gram negative bacteria. In this alternate Gram staining procedure smears are made on glass slides, air dried and heat fixed, whereafter smears are covered with freshly thawed lectin solution for 30 s and then gently rinsed with phosphate buffer. Cover slips are then placed on the wet slides. When analyzed in a fluorescence microscope (488 nm), Gram positive bacteria fluoresce bright yellow-green, whereas Gram negative bacteria do not fluoresce.

In contrast to preparation of cells on slides, however, flow cytometry is based upon analysis of cells in suspension. In order to develop the alternate Gram staining procedure described above for cells in suspension, effort has been directed towards development of preparation procedures leaving the Gram positive bacterial wall with a high affinity towards the lectin despite the lack of the slide fixation procedure. Preparations of cells on slides for analysis by microscopy have proved successful, and our work with cells in suspension is in progress.

Rapid bacterial antibiotic susceptibility testing by means of flow cytometry.

In addition to efforts directed towards development of flow cytometry based procedures for identification of large groups of bacteria based upon binding of fluorescence-labeled lectin to specific bacterial wall components, we have been aiming to investigate the feasability of this method for antibiotic susceptibility testing in bacteria. So far, our work has been based upon experiments with beta-lactam antibiotics and quinolones. It is well established that effects on light scattering and fluorescence parameters may be detected by means of flow cytometry within minutes after drug addition. However, most articles published within this field have been pilot studies based upon drug concentrations far exceeding the ones of clinical interest. In the present studies we have therefore focused on drug concentrations within the range of clinical relevance.

The response of exponentially growing *E.coli* cells to antibiotics was analyzed by flow cytometry and spectrophotometry as well as by a conventional MIC (minimum inhibitory concentration) assay. Cells were incubated with drugs during exponential growth and sampled every 20 min. Concentrations of ampicillin and mecillinam ranged from 1/4 MIC to 4 MIC and 50 MIC respectively and from 1/2 to 8 MIC for ceftazidime and ciprofloxacin. Samples for flow cytometry were stained with DNA specific dyes after fixation in ethanol. It should be noted that much effort has been directed towards development of cellular preparation procedures not including fixation in ethanol, since ethanol-fixation is time-consuming, requires sentrifugation, and leads to loss of cells. Thus, parallel samples were permeabilized by means of cold shock,

[redacted] Inhibition of cell proliferation was detected by measurements of optical density as well as flow cytometry-based measurements of fluorescence, forward and high angle light scattering, and cell numbers. By means of flow cytometry, increases in cellular DNA content and light scattering were

detected within 30 mins after addition of 1 MIC ampicillin and mecillinam, respectively, Figs. 1 to 4. For ceftazidime and ciprofloxacin similar results were obtained even for 1/2 MIC of the drugs. For all drugs, the cold-shock preparation procedure increased accuracy of cell counts as compared to the fixation procedure. Effects on optical density were evident from about 80 mins. The results suggest that flow cytometry may have a potential as a rapid, automated and quantitative technique for bacterial susceptibility testing of antibiotics within concentrations of clinical interest.

Figure legend (Dette er kopiert direkte fra den artikkelen som er til review, huff da.)

Fig. 1 shows **a** optical density (OD), **b** fluorescence (FL), **c** low angle light scattering (LS), **d** high angle LS, and **e** relative cell number as a function of time with mecillinam. FL is a measure of cellular DNA content, while LS parameters reflect size and refractive index, respectively. The data in **b** - **e** were obtained by flow cytometric measurements of single cells, while the data in **a** were obtained by measurements of the cell culture OD. The values in **a-d** were median values calculated from three parallel experiments, and the range is hence given.

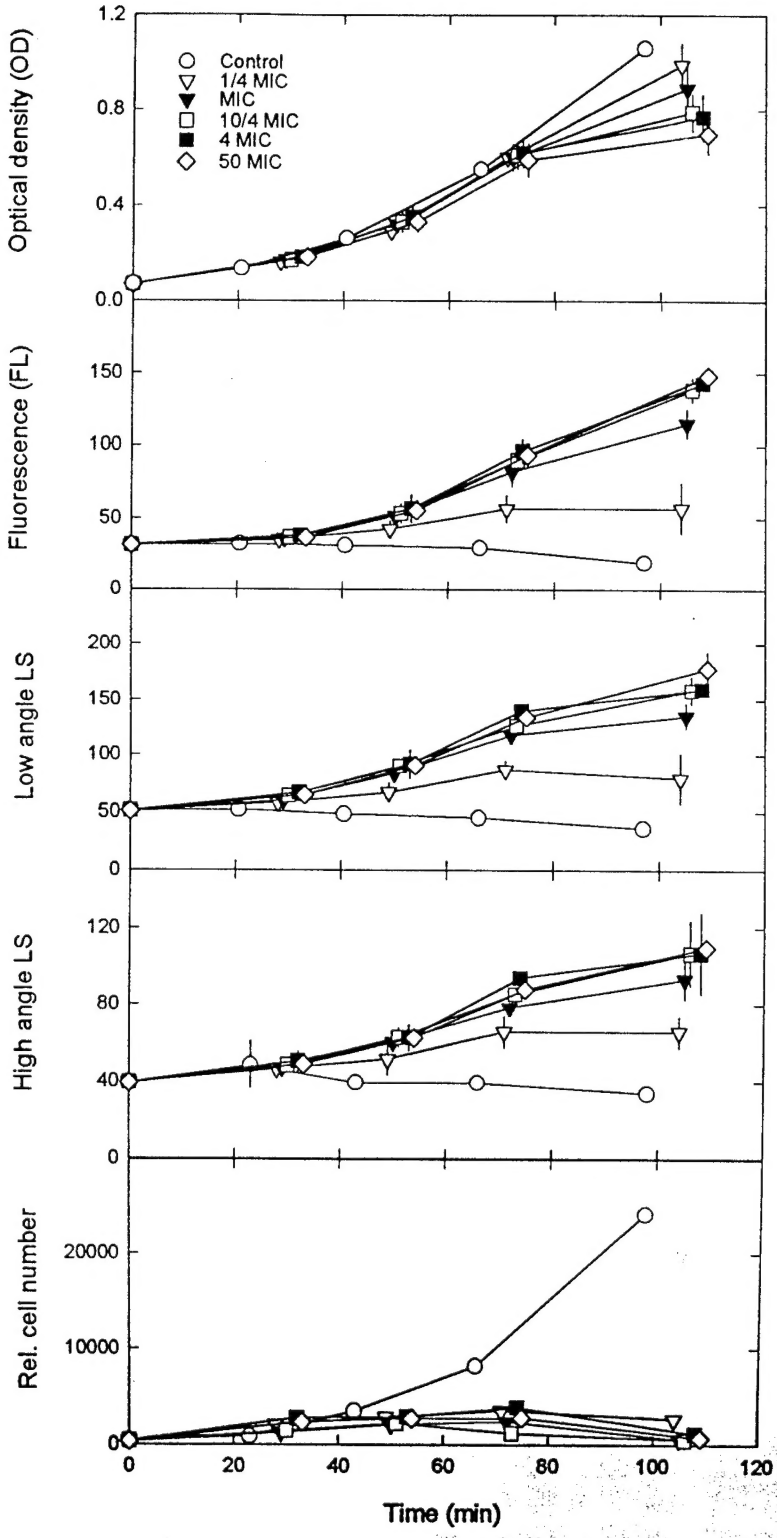
Fig. 2. Fluorescence (FL) (**a**), low angle light scattering (LS) (**b**), and large angle LS (**c**) histograms of samples taken 30, 50, 70, 105 min after addition of 1 MIC mecillinam.

Fig. 3 shows **a** optical density (OD), **b** fluorescence (FL), **c** low angle ligh scattering (LS), **d** high angle LS, and **e** relative cell number plotted versus time in cells exposed to ampicillin. For details, see Fig. 1. The values in **a-d** were median values calculated from three parallel experiments, and the range is hence given. The data in **e** were based upon

results from one single experiment, it should be noted, however, that similar results were obtained also in several parallel experiments.

Fig. 4. Fluorescence (FL) (**a**), low angle light scattering (LS) (**b**), and large angle LS (**c**) histograms of samples taken 25, 45, 70, and 100 min after addition of 1 MIC mecillinam.

Fig. 1


Optical density (OD)
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MIC
10/4 MIC
4 MIC
50 MIC
1.2
0.8
0.4
0.0
a
Fluorescence (FL)
150
100
50
0
b
Low angle LS
200
150
100
50
0
c
High angle LS
120
80
40
0
d
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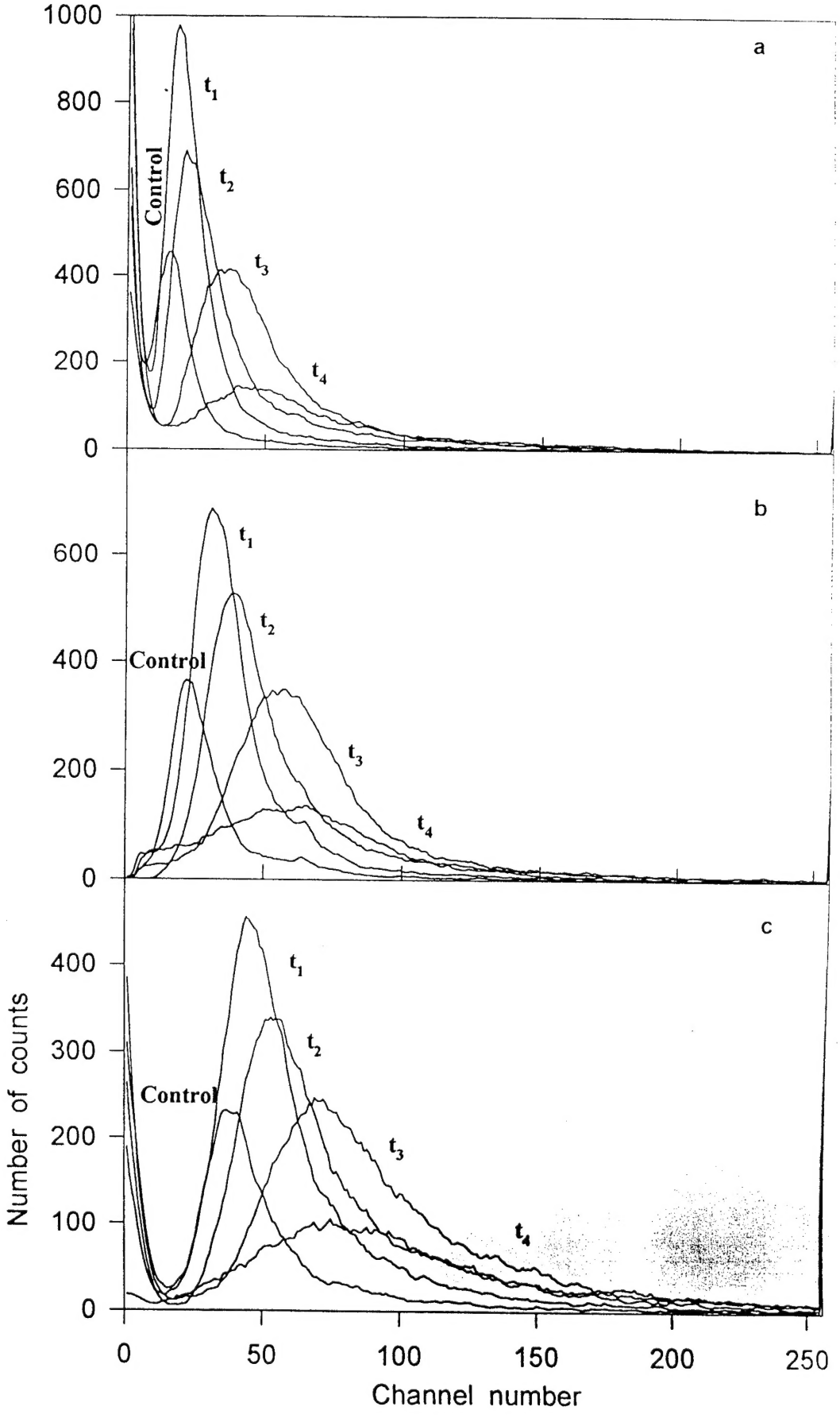
10000
0
e
0
20
40
60
80
100
120
Time (min)

Fig. 2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b
c
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Control
Control
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t_2
t_3
t_4
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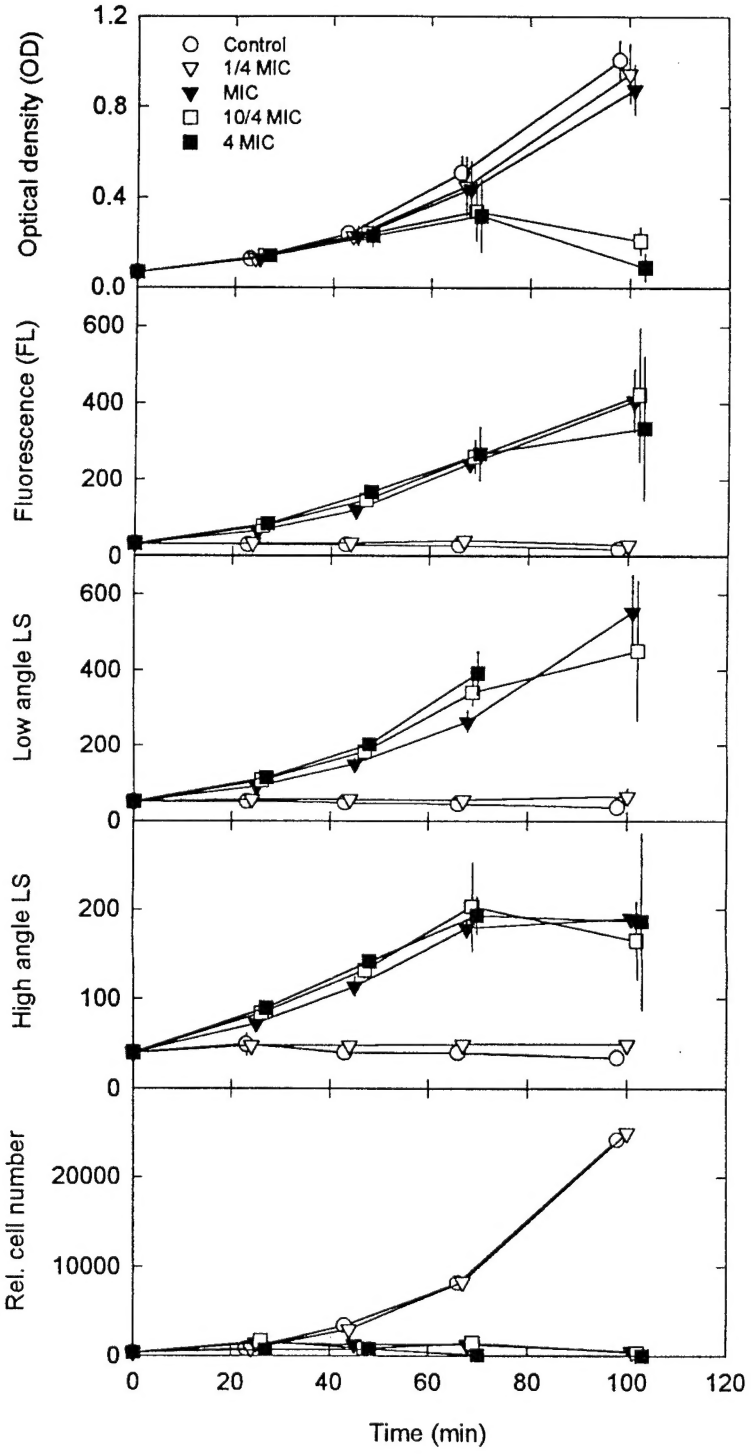
800
600
400
200
0
600
400
200
0
400
300
200
100
0
0
50
100
150
200
250
Number of counts
Channel number

Fig. 3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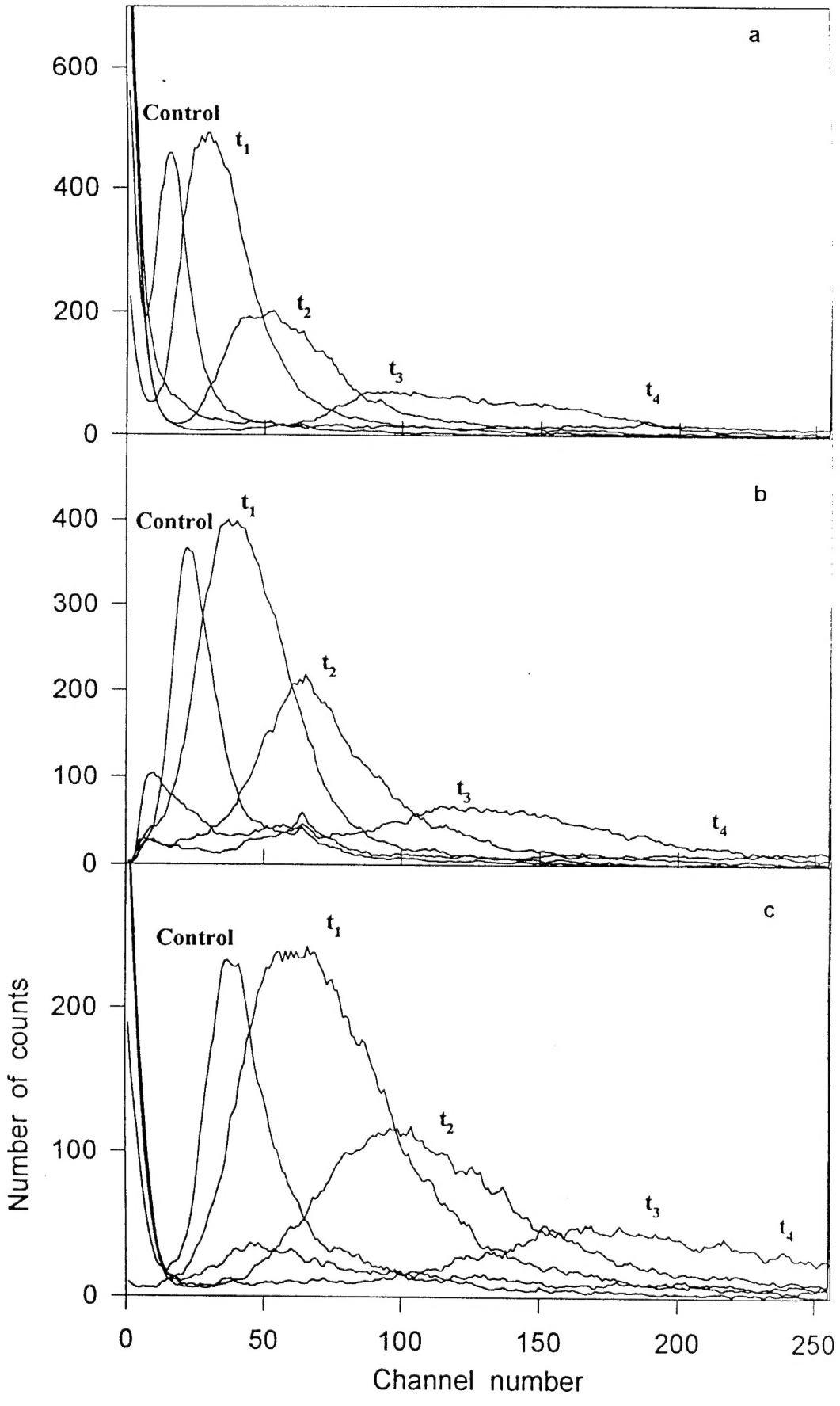
0.8
0.4
0.0
Control
1/4 MIC
MIC
10/4 MIC
4 MIC
a
Fluorescence (FL)
600
400
200
0
b
Low angle LS
600
400
200
0
c
High angle LS
200
100
0
d
Rel. cell number
20000
10000
0
e
0
20
40
60
80
100
120
Time (min)

Fig. 4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400
200
0
Control
t1
t2
t3
t4
b
400
300
200
100
0
Control
t1
t2
t3
t4
c
200
100
0
Control
t1
t2
t3
t4
Number of counts
0
50
100
150
200
250
Channel number